REMARKS / ARGUMENTS

By the present amendment, claim 38 has been amended as described below. The amendment to the claim have been made without prejudice and without acquiescing to any of the Examiner's rejections. No new matter has been entered by the present amendment and its entry is respectfully requested.

The Applicant withdraws claims 5, 10-21, 25, 27-28, 32-33, 36-37, 39-46, 48-52.
Claims 1-4, 6-9, 22-24, 29-31, 34-35, 38, 47 are respectfully submitted for examination.

The office action dated December 8, 2010 has been carefully considered. It is believed that the following comments represent a complete response to the Examiner's rejections and place the present application in condition for allowance. Reconsideration is respectfully requested.

35 USC 103(a)

The Examiner rejected claims 1-2, 5-6, 8-9, 22-23, 32, 34-35 and 38-40 under 35 USC 103(a) as being unpatentable over US Patent Publication No. 2004/0142488 to Gierde et al. ("Gierde") in view of US Patent Publication No. 7,379,820 to Sukits et al. ("Sukits"). The Examiner alleges that Gierde teaches steps (a) to (c) of present claim 1 at paragraphs [137] and [195-201] and that Sukits teaches separating protein pairs by using NaCl. at Column 18, lines 30-38. The Examiner is of the opinion that one of ordinary skill in the art would have found it obvious to modify Gierde's method to investigate protein-protein interactions in a multi-protein complex using the affinity chromatography assay and that by combining Gierde and Sukits, Gierde's method would be modified to include a NaCl elution step on an immobilized protein-protein complex. On page 3 the Examiner cites the disclosure at paragraphs [195-202] of US Patent Publication No. 2004/0142488 to Gierde et al. ("Gierde") as disclosing that the biomolecule is a multi-protein complex and that one or more constituents of the multi-protein complex can be recovered. In addition, the Examiner states that Gierde [2000] is supported by priority document U.S. Patent App. 60/396,595, page 95.

Applicant already submited that Gierde [195-202] is not citable against the present application. Gierde was published on July 22, 2004 and filed on January 8, 2004. The present application has an international filing date of July 30, 2004 and claims priority dating back to US Provisional 60/494,811 filed on August 14, 2003, which predates the Gierde application. Gierde claims priority to other previously filed applications, US 10/620,155 filed July 14, 2003, US 60/465,606 filed on April 25, 2003 and US 60/396,595 filed July 15, 2002. Applicant has reviewed these three Gierde priority documents and respectfully submits that these three documents, and particularly US 60/396,595 filed July 15, 2002, pp72-73 of the 199 page document, do not provide support for paragraphs [195-202] of the presently cited Gierde application. The Examiner agreed with our arguments but in his response from December 8, 2010 Examiner states that support for paragraphs [195-202] of "Gierde" can be found in another place, namely on p95 of the 199 page document, US 60/396,595, which describes an antibody-antigen complex that is immobilized on an affinity column by biotin-streptavidin bond and wherein the antigen is eluted but the antibody remains immobilized. Applicants respectfully disagree for the reasons that follow.

(I) Biotinylating of the antibody is used in order to increase the specificity of the immobilization and purification of the antigen. Briefly, Biotin (Vitamin H or Vitamin B7) is covalently coupled to primary amines (lysines) of the immunoglobulin, i.e. the antibody, and after binding the biotinylated antibody to the antigen, the biotinylated antibody is bound strongly to streptavidin column. (The avidin-biotin complex is the strongest known non-covalent interaction, $K_d = 10^{-15}$M, between a protein and ligand. Similarly, streptavidin-biotin complex has $K_d = 10^{-14}$M to 10^{-15}M). After removing unbound substances, the antigen is released from the biotinylated antibody under conditions that do not affect the bond between the biotinylated antibody and the streptavidin column. However, although biotinylation greatly increases the efficiency of the antigen isolation, biotinylated antibody simply constitutes an additional artificial link, i.e. formed in vitro during the experiment, between the antibody and the affinity column. Thus, purification utilizing biotinylated antibodies constitutes an affinity chromatography method which is different from the present method. Indeed, p95 of US 60/396,595 belongs to chapter "Affinity Chromatography, Principles and Methods". At the end of the Affinity purification utilizing biotinylated antibody, the antigen, i.e. the second ligand that has been expressed in vivo and has

been attached to the affinity column, is removed from the affinity column. This teaches away from the present invention. See the attached FIG. 13 from the instant patent application and "Comparison between the Affinity purification utilizing biotinylated antibody and the present invention" illustrating the differences.

(II) The Applicant respectfully objects that the multi-protein complexes described in Gierde [195-202] are substantially different from the complex biotinylated antibody-antigen described in U.S. Patent App. 60/396,595, page 95 for the following reasons:

1 - Before suggesting partial disruption of the multi-protein complexes in Gierde [0200], Gierde [0196] defines Multi-Protein Complexes as "extracted from a biological sample using a sample solution and extraction conditions that stabilize the association between the constituents of the complex." Similarly, claims 1-4 of the present invention clearly define the complex between the second ligand and first ligand as formed in vivo, i.e. before the extraction from a biological sample.

In contrast, affinity purification utilizing biotinylated antibody includes formation de novo, i.e. during the course of the experiment, after extraction of the proteins from a biological sample, of two protein-protein bonds, i.e. (1) the bond between the antigen and the biotinylated antibody and (2) the bond between the affinity column and the biotinylated antibody. Therefore, the complex containing the biotinylated antibody and the antigen is outside of the definition of "Multi-Protein Complexes" in Gierde [0196], i.e. "extracted from a biological sample".

2 - Antibodies must be obtained from the exogenous organism, i.e. organism different from the one that expresses the antigen, in order to avoid cross reaction with proteins, other than the antigen. Therefore, the multiprotein complex as defined by Gierde in [0196] is substantially different from the complex consisting of biotinylated antibody and antigen.

3 - Gierde [0197] states "In some embodiments, multi-protein complex is adsorbed to the extraction surface and desorbed under conditions such that the integrity of the complex is retained throughout." However, it is impossible to desorb the biotinylated antibody from the streptavidin column without disrupting the bond between the biotinylated antibody and the

antigen because of the harsh conditions, usually 8 M guanidine-HCl, pH 1.5. See priority document U.S. Patent App. 60/396,595, page 95 and 96. Buffers used for disrupting the bond between the biotinylated antibody and the antigen, apply milder conditions: (a) 50 mM ammonium acetate, 0.5 M NaCl, pH 4.0; or (b) 0.1 M glycine-HCl, pH 2.2. See priority document U.S. Patent App. 60/396,595, page 95 and 96. This proves that the multiprotein complexes of Gierde 0196] are fundamentally different that the complex consisting of biotinylated antibody and antigen.

Moreover, if the antigen was part of in vivo formed protein complex, the conditions that these two buffers, i.e. 50 mM ammonium acetate, 0.5 M NaCl, pH 4.0 or 0.1 M glycine-HCl, pH 2.2, would apply are harsh enough to destroy any in vivo formed protein bonds. This contradicts Gierde [0197] that states that conditions must be "such that the integrity of the complex is retained throughout."

In addition, U.S. Patent App. 60/396,595, page 95 and 96, describe a Binding buffer, i.e., the buffer providing conditions under which the binding of the antibody to the antigen and the following immobilization of the antibody to an affinity column is performed, containing 50 mM ammonium carbonate, 0.5 M NaCl, pH 10. This binding buffer is also harsh enough to destroy any in vivo formed protein bonds. This contradicts Gierde [0197],see above.

In view of the foregoing, the Applicant respectfully submits that the disclosure in Gierde [2000] is not supported by priority document U.S. Patent App. 60/396,595, page 95 and therefore is not citable against the present invention. The Applicant reiterates that Gierde [2000-2001], published on July 22, 2004 and filed on January 8, 2004, was conceived after the Applicant reduced the present invention to practice, i.e. isolated the first ligands and identified them, in US Provisional 60/494,811 filed on August 14, 2003.

Even if Gierde [195-201] were citable, which we disagree with, Gierde [0137 and 0195-0201] and Sukits [column 18, lines 30-38] do not teach or suggest that the second ligand (the protein that is immobilized on the affinity matrix) must remain immobilized on the affinity column during the elution of the first ligand. As described in the present application, desorption of the second ligand must be avoided, because otherwise a severe dynamic range problem would be encountered, which would make the identification of the first ligand impossible. The mere

statement by Gierde at paragraph [0201] that "some subset of the complex is released while the rest remains adsorbed", is very vague and does not provide any guidance that would lead to the presently claimed method. In this regard, neither Gierde [0137 and 0195-0201] nor Sukits [column 18, lines 30-38] teach or suggest that the elution agent should be chosen in such a way that it favors the separation of the first and second ligand, while not affecting the bond between the second ligand and the affinity matrix. In fact, Gierde [0201] proposes two approaches that teach away from the present invention: "For example, by decreasing the polarity of a desorption solvent hydrophobic interactions will be weakened-inclusion of reducing agent (such as mercaptoethanol or dithiothrietol) will disrupt disulfide bridges." Moreover, these two approaches would not accomplish the present invention for the following reasons:

(a) - Decreasing the polarity of a desorption agent would weaken not only the hydrophobic interactions within the protein complex but the bond between the affinity matrix and the immobilized second ligand, as well. (Hydrophobic forces are present in every protein-protein association). Thus, separation of the first ligand from the immobilized second ligand as described in the present application would not be accomplished.

(b) - Inclusion of reducing agent would weaken indiscriminately the disulfide bridges and would affect the permanent protein-protein association to a much bigger extent than the transient (predominantly electrostatic) interactions. Thus the dynamic range problem would not be avoided. Moreover, inclusion of reducing agent would weaken not only the interactions within the protein complex but the bond between the affinity matrix and the immobilized second ligand, as well.

The Applicants were first to propose and demonstrate the usefulness and desirability of separating the first ligand from the immobilized second ligand, i.e. the elimination of the dynamic range problem [0009-0015 and 0077- FIG. 21] in the present application. More importantly, this application is the first to propose the exact way to accomplish separation of the first ligand from the immobilized second ligand. Also, see below the arguments for non obviousness with regard to Rigaut in view of Sukits and Gierde.

In view of the foregoing, Applicant respectfully submits that claims 1-2, 6, 22-23, 34-35 and 38 are inventive over Gierde in view of Sukits.

On page 7 the Examiner rejected claims 3-4, and 10-21 under 35 USC 103(a) as being obvious having regard to Rigaut et al. ("Rigaut", Nature Biotechnology (1999) 17:1030-1032) in view of Sukits and Gierde. The Examiner alleges that it would be obvious to modify Rigaut's method to investigate protein-protein interactions in multi-protein complexes using electrostatic elution. According to the Examiner, the person skilled in the art would modify Rigaut's method to include a NaCl elution step on the multi-protein complex and that the person skilled in the art would be motivated to do so based on Gierde's indication that the nature of multi-protein complexes can be analyzed by eluting individual components. Applicants respectfully point out that as of the priority date of the present application, Gierde did not disclose that the nature of multi-protein complexes can be analyzed by eluting individual components. Such an indication was only added to Gierde after the present application's priority date. In view of the foregoing, the Gierde sections relied upon by the Examiner are not citable against the present application and thus, we respectfully submit that there is no motivation to modify Rigaut's method in view of Sukits.

Rigaut does not teach that the first and second ligands can associate through electrostatic forces or suggest that the second ligand (the protein that is immobilized on the affinity matrix) must remain immobilized on the affinity column during the elution of the first ligand. As described in the present application, desorption of the second ligand must be avoided, see paragraph [0354] of the present application. Otherwise, a severe dynamic range problem will be encountered, which will make the identification of the first ligand impossible. See [0005-0015] and [0077- FIG. 21] of the present application. Thus one of ordinary skill in the art following the teachings of Rigaut would try to isolate the first ligand and second ligand together and present the result as a strong proof for their interaction/association. This teaches away from the present invention.

Prior to the present application, a person skilled in the art would not be motivated to disrupt electrostatic interactions from the immobilized multi-protein complex described in Rigaut based on the disclosure of Sukits. Rigaut do not suggest the desirability, and thus the obvious nature, of step 1(d). The inventors of the claimed invention were the first to suggest and demonstrate that elimination of the dynamic range problem by separating the substoichiometrically interacting

proteins (i.e. first ligand) from the immobilized fusion protein (i.e. second ligand that remains immobilized) is possible and desirable (see patent application [0004-0015] and [0057-0067]). The cause for the failure to detect substoichiometrically interacting proteins was not obvious at the priority date of the invention. For example, the substoichiometric interactors (i.e. first ligands) could have just been absent after immobilizing the protein of interest (i.e. second ligand) on affinity matrix or, they could have been present at amounts that do not allow their proper analysis and identification.

The applicants were the first to identify the cause for this failure, i.e. high dynamic range, and have designed a method which solves the problem. Three non-obvious concepts are incorporated in the invention:

- transient interactors (i.e. first ligands) are present after the immobilization of the protein of interest (i.e. second ligand) on the affinity matrix but they are not detected because of the low stoichiometry (high dynamic range);
- transient interactors have to be separated from the high abundance affinity tagged protein and analyzed separately; in addition, the separation should not lead to separation of any other associated high abundance protein from the affinity tagged protein.
- the separation of in vivo formed transient protein complexes can be achieved by increasing the ionic strength;

The Applicant respectfully submits that the priority documents mentioned by the Examiner, i.e. Gierde, Sukits and Rigaut, do not suggest the usefulness and desirability of separating the first ligand, which has been associated in vivo with the second ligand before the start of the experiment, from the second ligand which remains bound to the affinity column. The Applicants were the first to propose and demonstrate that an in vivo formed protein complex can be immobilized on an affinity matrix in such a way that would allow for the selective separation of the proteins (i.e. first ligands) that interact with the immobilized affinity tagged protein (i.e. second ligand) while the latter remains immobilized. Prior to the present application, a person skilled in the art would not expect to be able to separate transiently interacting substoichiometric proteins from permanent protein members of such an *in vivo* formed complex. One skilled in the art would try to perform the present invention only after following the teaching in [0219] of this

application that states "Permanent protein complexes are held together mainly by hydrophobic forces and transient ligand complexes are held together in large part by electrostatic attractions".

In view of the above, Applicants respectfully submit that claims 3-4 are inventive over Rigaut in view of Sukits and Gierde.

On p16 Examiner argues that since Sukits provides evidence that NaCl can separate proteins electrostatically joined in vitro and that are found to interact in vivo, the skilled artisan would have found it obvious that an in vivo formed protein complex can be disrupted by applying NaCl. Examiner argues that the Applicant needs to provide evidence that separation of in vivo formed protein pair necessarily requires a different separation approach from in vivo joined proteins.

Applicants respectfully submit that two ligands can be associated in vivo by electrostatic bonds but it does not necessarily mean that they can be separated from one another by increasing the ionic strength of the medium. The evidence demanded by the Examiner is in "Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution" by Cramer P, Bushnell D, Kornberg R. (Science, 2001, http://www.sciencemag.org/cgi/reprint/292/5523/1863.pdf, previously submitted), Table 2 - "Subunit interactions", Rpb1 forms 6 salt bonds, i.e. electrostatic bonds, with Rpb2, 5 salt bonds with Rpb5, and three salt bonds with Rpb6 and Rpb8. In total, there are 45 electrostatic bonds between the 12 subunits, i.e. Rpb1, Rpb2...Rpb11, Rpb12, of the permanent protein complex RNA polymerase II. After immobilizing the RNAP II complexes on affinity matrix by affinity tagged Rpb1, the other 11 subunits of the core RNA Polymerase II complex are present at approximately the same molar amount as Rpb1. This amount is much higher (with at least an order of magnitude) than the amount of the transcription factors. Yet, the 11 subunits of the core RNA Polymerase II complex (other than the immobilized Rpb1), are not eluted upon increasing the ionic strength (see the results in Figures 3, 4, 6, 7, 8, 10, 11, 12). If one of ordinary skill in the art follows the teachings of Sukits, Rigaut and Gierde, he would expect Rpb2, Rpb5, Rpb6 and Rpb8 to be eluted/ desorbed upon increasing the ionic strength.

Although it is probably not necessary to provide explanation, Applicant believes there are at least two reasons: (a) the electrostatic bonds might be inaccessible to the small ions (resulting from

the increased ionic strength) and/or (b) beside the electrostatic bonds, the two ligands can be associated by other bonds, i.e. hydrophobic bonds, and even if the electrostatic bonds are disrupted, the two ligands can remain bound to each other. Yet, in both cases the association between the two ligands can be most appropriately described as "associated by electrostatic bonds" because the energy of the electrostatic bonds, especially the Coulomb forces, is much bigger than the energy of the non-electrostatic bonds.

The fact that Rpb2, Rpb5, Rpb6 and Rpb8 remain bound to the immobilized Rpb1, despite the separation of 50 other proteins, argues strongly that the present invention is not obvious. (In other words, if step 1(d) is obvious, i.e. that two ligands that associate by electrostatic bonds can be separated be destroying the electrostatic bonds, why are not the other 11 subunits of the core RNA Polymerase II complex eluted and detected upon increasing the ionic strength?)

Further, in step 1(c) of the presently claimed method, prior to salt elution, the only *de novo* formed protein-protein interaction is the one between the affinity matrix and the second ligand. Therefore, the prior art of Gierde and Sukits teaches one of ordinary skill in the art that upon increasing the salt concentration the most obvious result would be the separation of the second ligand from the affinity matrix (with or without the separation of the second ligand from the first ligand), which teaches away from the presently claimed method.

Regarding claim 34, on page 6 the Examiner opines that NaCl has the "capability" to separate one protein from the other. Applicant respectfully submits that NaCl is not a biomolecule and that NaCl affects non-selectively many protein-protein pairs as shown in Figures 3-12, i.e. around 70 different proteins were separated from the immobilized second ligand by using one agent, i.e. KCl. Similarly, Sukits describes non-selective separation of several protein pairs, i.e. RIP DD- TRADD, FAS DD -FADD DD and TNFR-1 DD with TRADD DD by increasing the ionic strength with one chemical agent – NaCl. In contrast, claim 34 clearly states that the "biomolecule is identified as a drug or pre-drug by its capability to separate **selectively** the first ligand from the second ligand and/or affect **selectively** the separation of the first ligand from the second ligand" (Emphasis added). Paragraphs [0249 - 0261] of Description of the invention describe the difference between the non-selective effect of increasing the ionic strength on any electrostatically bound protein-protein pair and the selective effect of biomolecule on a specific

protein-protein pair. Please find attached Figure 19 illustrating the difference between selective and non-selective separation of protein pair. In addition, claim 34 defines the biomolecule as capable to separate two proteins at "the change of the concentration of the chemical or biomolecule is below 30 mM".

Regarding claim 38, on page 7 the Examiner opines that since the object of the combination of Gierde and Sukits is to separate the protein constituents, one of ordinary skills in the art would have found it obvious to include an electrostatic charge identical to a mutation (species (b) in the claim). Applicant objects that combining Gierde and Sukits, i.e. increasing the ionic strength, would lead to non-selective effect on any electrostatically associated proteins and reiterates the differences between selective disruption of a particular protein-protein pair by applying a specific biomolecule and non-selective separation of any electrostatically bound protein-protein pair by increasing the ionic strength. See the previous paragraph. In addition, Applicant amends claim 38 in order to narrow the definition of the claimed chemical or biomolecule so that claim 38 states "all of the following features" instead of "one or more of the following features". In addition, the Applicant reiterates that this application has a priority over Gierde.

In view of the above, Applicant respectfully submits that claims 34 and 38 are inventive over Gierde in view of Sukits.

On page 12 the Examiner rejected claim 24 under 35 USC 103(a) as being unpatentable over Rigaut in view of Sukits and Gierde as applied to claims 1 and 22-23 above, and further in view of US Patent No. 5,007,934 to Stone and US Patent No. 5,849,885 to Nuyens et al ("Nuyens"). As discussed above, paragraphs [195-201] of Gierde do not pre-date the present application's priority date and thus are not citable against the present application. Rigaut in view of Gierde and Sukits further does not teach or suggest step 1(d) separating the first ligand from the immobilized second ligand, which remains bound to the affinity matrix during the separation. Thus, neither Stone nor Nuyens correct for the deficiencies discussed above of Rigaut in view of Gierde and Sukits.

On p17 Examiner argues that since Gierde and Rigaut describe the same affinity tags, that are claimed in the invention, i.e. GST,CBP, MBP and Staphylococcus aureus Protein A, as capable of immobilizing the second ligand, it would necessarily allow for elution of the first ligand without removing the second ligand if ionic strength is increased according to Sukits. The applicant objects that immobilization is performed at low salt concentration, i.e. 150 mM KCl, and elutions are performed at higher salt elutions which disrupt not only the electrostatic bonds between the first and second ligand but the electrostatic bonds between the affinity column and the affinity tagged second ligand. See [0301] of this application where 0.7 M potassium acetate is used to separate the first ligand from the immobilized second ligand. See the above example of the ubiquity of electrostatic bonds between proteins. Applicants respectfully submit that for the presently claimed methods, not only is the bond between the first ligand and the second ligand a protein-protein bond, but the bond between the second ligand and the affinity matrix is also a protein-protein bond.

As a further evidence for the non-obviousness of the invention, the Applicant respectfully points out that the present invention solves an outstanding and long-recognized problem in the field of detecting protein-protein interactions, i.e. high dynamic range problem. This is demonstrated by the huge difference between the results obtained by using the method of the present invention and the prior art.

As an example, Gavin et al. Nature (2002), previously submitted, used the method of Rigaut on a large scale (1,739 protein purifications) and, in particular, performed 3 protein purifications by using an affinity tag fused to 3 different subunits (Rpb3, Rpb7 and Rpb9) of RNA polymerase II which is a permanent complex of twelve subunits (Rpb1-Rpb12). Since the purpose of the method of "Rigaut" is to isolate the entire in vivo formed protein complex, i.e. second ligand and first ligand(s), they detect only 7 proteins that interact with the permanent complex (See Supplementary table S1). By contrast, by using the method of our invention, we were able to identify more than 70 additional interacting proteins (paragraph [0357] of this patent application and Figures 3-12). Moreover, Gavin et al. Nature (2002) demonstrates that a team of 38 skilled scientists performed 1,739 protein purification but failed to realize that separating of the first ligand from the immobilized second ligand would greatly improve the final result. This argues for the non-obviousness of the invention.

The same failure is demonstrated in Krogan et al. Nature (2006), http://www.ncbi.nlm.nih.gov/pubmed/16554755, where 52 scientists processed 4,562 tagged proteins, i.e. second ligands, in order to detect the interacting proteins, i.e. first ligands, and again did not realize the desirability and usefulness of separating the first ligand from the immobilized second ligand. The Applicants were the first to propose and demonstrate that an in vivo formed protein complex can be immobilized on an affinity matrix in such a way that would allow for the selective separation of the proteins (i.e. first ligands) that had been associated with the immobilized affinity tagged protein (i.e. second ligand) while the latter remains immobilized.

Applicant respectfully request that the rejections under 35 USC 103(a) be withdrawn and submits that claims 1-4, 6-9, 22-24, 29-31, 34-35, 38, 47 are inventive.

In view of the foregoing comments and amendments, we respectfully submit that the application is in order for allowance. Should the Examiner deem it beneficial to discuss the application in greater detail, he is kindly requested to contact the undersigned by telephone at (519) 837-9427 at his convenience.

Respectfully submitted,

By _____

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